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Fine Mapping of the Giant Embryo Gene *GE2* in Rice

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ABSTRACT

Giant embryo rice enriched with micro-nutrients can promote nutrition level of rice, and breeding of giant embryo rice is an important task. A new giant embryo variety, Tainung 78 developed by mutagenesis breeding, exhibits similar phenotypes of agronomic traits but 3~5 fold large embryo size to those of its derived parent, Tainung 72. After linkage analysis of 46 F_2 individuals of TNG 78 × TCS 17, the mutated gene conferring giant embryo was coarsely mapped in the interval between *RM243* and *RM420* on the long arm of chromosome 7, which was different position from the *GE*, and was temporally named as *GE2*. By employing additional 87 F_2 individuals and 5 markers to fine map *GE2*, the chromosome segment encompassing *GE2* was confined to 170 kb and contained 24 candidate genes after annotation. High resolution mapping by using more individuals and markers and sequencing the candidate genes are subjected to touch the target gene. Once *GE2* isolated via this positional cloning strategy, we can elucidate the mechanism of *GE2* how it enlarges embryo size under gene function and regulation. In the meanwhile, the two flanking markers of *GE2*, *CH0709* and *CH0720*, are subjected to rice breeding programs to breed more

giant embryo varieties. Furthermore, we are attempting to pyramid *GE2* and the golden rice gene of Tainung 76, *GR1* to purple rice CNY941201, to promote the nutrition of rice.

Key words: Giant embryo, Genetic mapping, Marker-assisted breeding, Rice, Tainung 78 (TNG 78).

INTRODUCTION

Rice, domesticated 9000 years ago, is the most important crop and is cultivated between 55°N and 36°S latitudes in various environments, such as irrigated, rainfed and floodprone ecosystems. There are two cultivated rice species, *Oryza glaberrima* cultivated only in Africa and *Oryza sativa* with two major subspecies, *japonica* and *indica*, widely cultivated mostly in Asian and other parts of the world (Khush 1997). More than three billion people in the world rely on rice, providing 23 % of calories consumed by human (Khush 2003). On the other hand, about 90% of rice is produced in Asia (Fisher *et al*. 2000), providing up to 35~60% of the total calories consumed by Asians (Khush and Brar 2002).

The edible endosperm contains carbonhydrate with majority which provides daily calories; on the other hand, rice embryo contains several micronutrients such as fatty acid, vitamins B and E, nicotana, and so on. Giant embryo with enlarged embryo size can promote the nutrition of rice. The giant embryo mutant of Kinmaze, induced by NMU (N-methyl-N-nitrosourea), enlarged embryo size of 2~3 fold embryo and increased the total fatty acid from 2.6% to 3.91% (Satoh and Omura 1981; Sato and Iwata 1990). Giant embryo rice with enlarged embryos enriches gamma-amino butyric acid (GABA) content in pre-germinated seeds (Yutaka *et al*. 1994), which is good for decreasing cholesterol and increasing antioxidants in vivo (Lee *et al*. 2007).

Three QTLs affecting embryo length mapped on chromosomes 1, 2, and 3, and three QTLs affecting embryo width mapped on chromosomes 2, 8, and 10, respectively, were identified from a *japonica/indica* cross (Dong *et al*. 2003). *GE*, a giant embryo locus with four mutant alleles (*ge-2*, *ge-3*, *ge-4*, and *ge-5*) induced by NMU, was reported and mapped on chromosome 7. The giant embryo was the consequence of enlarged scutellum and degenerated endosperm (Hong *et al*. 1995; 1996). Six giant embryo lines with 2~5 fold enlarged embryo size were selected and breed from *japonica* Tainung 72 induced by NMU (Wu and Lur 2002), and one of six was entitled as Tainung 78. In this study, we mapped the giant embryo locus of Tainung 78 by linkage analysis of genotypes of the giant embryo and molecular markers from segregating F_2 populations. The flanking markers of the giant embryo locus can be implemented marker-assisted selection in rice breeding to breed more elite cultivars possessing enlarged embryo.

MATERIALS AND METHODS

Plant Materials

The panicles of 20 individuals, after 10 hours of self pollination, of *Oryza sativa* ssp. *japonica* cv. Tainung 72, TNG 72, were soaked in 1 mM N-methyl-N-nitrosourea (NMU) for 4 hours; rice panicle were washed by running water about 6 hours and were planted in greenhouse to harvest M1 seeds in the first crop season, 2002. Approximate 4000 M1 were grown in paddy rice field of Chiayi Agricultural Experiment Station, Chiayi, Taiwan. The M1 plants were self pollinated, and three seeds of each M1 hill were randomly picked and bulked together. Approximate 4000 M2 individuals were planted in field in the first crop season 2003 and were screened for the desirable phenotype, giant embryo. Six M3 lines exhibiting giant embryo were consequently selected to self and propagate by pedigree method. The embryo size of the selected giant embryo

mutant line, entitled Tainung 78 (TNG 78), is about 3~5 fold larger than that of normal TNG 72 seeds.

To achieve the goal of fine mapping the mutated gene conferring giant embryo, the giant embryo variety TNG 78 was crossed to ssp. *indica* cv.Taichung Sen 17 (TCS 17), and the F_1 progenies were selfed to obtain F_2 seeds. About 46 and 600 F_2 individuals for coarse and fine genetic mapping were planted in paddy rice field of Chiayi Agricultural Experiment Station in the second crop seasons of 2007 and 2008, respectively.

Genetic Mapping of the Giant Embryo Gene

Molecular Marker Assay

The rice genomic DNA extraction was adopted the procedures of Li *et al.* (1995) with modification for mini-preparation. Approximately 0.02~0.1 gram of fresh tissues of 6- ~ 8-week-old young seedlings was homogenized in 900 μl of extraction buffer(100mM Tris, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl; 1.25% SDS; fresh-made 0.38% of NaHSO3) at a frequency of 30 1/s for 2 min by TisseLyser (Qiagen, Germany). The homogenized leaf tissues were incubated at 65℃ for 30 min with twice gently inverted mix, and was subsequently added with 270 μl of 5 M KOAc, which was set on ice for 20 min. The supernatants were saved after centrifugation at 15,000 rpm for 10 min at 4℃, and 700 μl of ice-cold isopropanol was consequently added to precipitate DNA. DNA pellets were washed with 70% of ethanol, air dried, and dissolved in 100 μl of TE buffer.

Two types of public PCR-based markers, simple sequence repeat from Gramene (SSR; McCouch *et al.* 2002, IRGSP 2005) and sequence tagged site (STS) from Rice Genome Research Program, were first used to survey polymorphic markers between the parents, TNG 78 and TCS 17. If the linkage distances of two adjacent polymorphic markers were larger than 20 cM, we designed indel

markers, affixed by CH, SLS, and STS, based on blast genomic sequences of *japonica* cv Nipponbare version 5 against *indica* 9311. A polymorphic sequences with longer than 10 bp were selected and designed primers by using Primer3 on the basis of the flanking sequences of indels for PCR products of 100~300 bp after application. A total of 22 newly designed polymorphic markers were subjected to coarse genetic linkage analysis of *GE2* (Table 1).

PCR was performed in 25 μl of reactions containing 0.2 μ*M* of each primer, 200 μ*M* deoxyribonucleotides, 1× T*aq* buffer, 0.5 unit of GeneTaq DNA Polymerase (GenePure Tech, Taiwan), and 40-60 ng of rice genomic DNA. The PCR profile was: 94℃ for 5 min, followed by 35 cycles of 94℃ for 1 min, 55℃ for 1 min, 72℃ for 2 min, and finally by 5 min at 72℃ for the final extension (Biometra, German). PCR products subsequently were run on 2.5% agarose at 250V for 14~23 min, using FEBE electrophoresis system (Faster Easier Better Electrophoresis, Biokeystone, California, USA).

Genetic Mapping of the Giant Embryo Gene

A total of 84 DNA makers including 47 SSR, 15 STS, and 22 indel markers were applied to the F_2 segregating population of 46 F_2 individuals for coarse linkage mapping analysis. The genotype of the giant embryo gene of each F_2 individual was predicted by the segregation ratio of embryo size of its 50 seeds. The putative genotypes of giant embryo accompanied with genotypes of 84 markers were employed for linkage analysis, which was performed by the program MAPMAKER/EXP version 3.0 (Lander *et al*. 1987), using the Kosambi function (Kosambi 1944). The LOD threshold was fixed at 3.5, markers are unlinked while genetic distance more than 40 cM. For fine mapping, additional 87 F_2 individuals and five markers in the target region were applied to narrow down the chromosome segment encompassing the giant embryo gene.

Physical Mapping Analysis of the *GE2* Locus

To find out the physical position of *GE2*, the primer sequences of flanking markers were used as queries to search the *japonica* cv Nipponbare sequence database by BLASTN. The BAC/PAC contig encompassing were consequently identified, which was based on the rice pseudomolecules (release 5) based on the IRGSP minimum tilling path in representing the 12 rice chromosomes (IRGSP 2005) and was retrieved from Rice Genome Annotation (http://rice.plantbiology.msu.edu/)

RESULTS

Inheritance of Giant Embryo of TNG 78

The giant embryo variety, Tainung 78 (TNG 78), was derived from Tainung 72 induced by NMU and breed by pedigree selection. The agronomic traits of TNG 78, such as plant height, heading date, days to maturity, are similar to those of TNG 72. There is no significant difference in grain shape of grains and brown rice of TNG 72 and TNG 78, neither (Fig. 1). However, the embryo sizes of TNG 72 and TNG 78 are distinguishable that the embryo size of TNG 78 about 3~5 fold larger due to mutation (Fig. 1). The rice bran of TNG 78 including embryo contain enriched total of fatty acids, total of protein, minerals, amino acids, vitamin E, and other micronutrients (data not shown).

After six-year selection and purification, the maturity, morphologies and agronomic characters of TNG 78 resemble those of its derived parent, TNG 72, in field trials for the following years. TNG 72 exhibit enlarged embryo in every crop seasons, which implies that the inheritance of giant embryo of TNG 78 is stable. From the segregation ratio of normal to large embryo in the F_2 segregating population of TNG 78 × TCS 17 indicated the mutated allele resulting enlarged

Table 1. The newly designed polymorphic markers between Tainung 78 and Taichung Sen 17.

Primer	Chr	Position[a]	Forward sequence	Reverse sequence
STS322	1	10.9	AAGCTTTGACTTGTATTGCAC	TTTGTATCATGTTCCCAATTC
STS326	1	55.7	GCTCAGTATATTACGGTGGAA	GGGAAGTAAATTGAATTGGTT
STS420	1	157.6	ATTACCGACAGCAATAGTTCA	CACAGGTCATACACCATCTTT
STS307	2	118.1	GGCTGTTCCTACTTCCTAGTC	GCCTTATCCTGTACTTACACC
STS331	3	91.1	TGGGAGTGACAACTCATCTAC	ACACCAGCAAATCAGATACAT
SLS178	3	115.6	AGAAGAGTTACCTCCATCCT	AGGCTTCAAGTTATTGCTAA
SLS189	4	28.6	ATCAGAATATTCGGGAAAAG	TTGTATACTCATTATGTAAATGGA
STS332	4	97.4	ACAAACAGTTGTATCACTGGG	GAGAGCTAACGTCCTTGATTT
STS344	4	108.2	GTCGAGAGCAACACAATACA	TTAGATCATGATTCGGAAATG
STS230	5	67.5	TATATGGATCATCATGTGCAA	TCACAAACATCTGTGTGCAG
STS358	6	96.5	CTCATGACCCTCATAGAGCTT	TTAAGATGACATTAAATCACAC
SLS164	7	41.7	CTGCATATTTTCCCCTATTA	GGACAAGGCACTAATACAGT
CH0866	8	0.5	CTCCTTTCCCAATCTTACCT	GCGCATGCAGTATTATGTTA
SLS182	8	21.6-25.2	ATCCTGACCTCTTGTTCTAC	TTAACATAGAAGACCATACGC
SLS188	8	92.2	GGCATCAGTAAGACACAATA	AATGAATCTGTCTAGATTGG
CH0862	8	105.7-106.1	GAAGACGAGTGAGGTCAGAA	TCCAATAAAACTGAGGCTGT
SLS506	9	45.2-49.3	ATCTCTCTAATCTTGCTGGCT	TCCGTAACCCAAATAAACATA
SLS510	9	58.3-60.8	TCAATTTGTGGGTTAGGTTTA	AACTGTGATTATCAACACGC
CH1105	11	2.5-2.8	TCGTTTCCTTCAAAACCTTA	TTCGGTTGAACTGATAAATGT
CH1106	11	57.3	TGTTCTCTAGGGGAACAAAA	ATGCCTTTTTCGTAGGTGTA
SLS173	11	91.4	ACCCCTACCTCTACTAGTGC	CGGTTTGGGTGATAATATAG
SLS167	12	39.4	GCAATAAAAACATAAAAGCA	TAAAAATATGCTGAGCAGTC

a The linkage map position of the markers are referred to IRGSP after blasting its primer sequences against BACs.

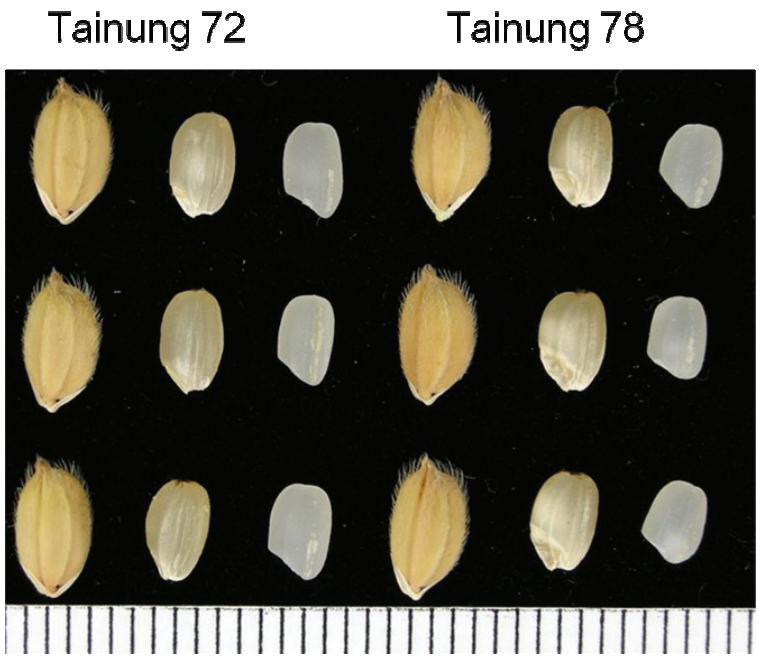

Fig. 1. The morphologies of seeds, brown rice and polished grains of TNG 72 (left) and TNG 78 (right).

Coarse Genetic Mapping of the *Giant Embryo 2*

A total of 84 PCR based markers, including 47 SSRs, 15 STS, and 22 indels, were subjected for coarse genetic mapping *Giant Embryo2, GE2*. Exclude chromosome 12 with one marker, the average interval of genetic distances of each chromosome ranged from 10.3 cM to 24.07 cM, with an average of 17.41 cM, based on the marker position aligned to the rice high-density linkage map published by Harushima *et al*. (1998) (Table 2).

Table 2. The markers employed to construct linkage analysis of *GE2*.

Chr	Genetic marker				Average genetic distance (cM)
	SSR	STS	Indel	subtotal	
1	5	3	3	11	20.41
2	9	0	1	10	17.09
3	4	3	2	9	15.12
4	3	1	3	7	17.35
5	4	2	1	7	14.80
6	6	0	1	7	17.71
7	5	1	1	7	24.07
8	3	1	4	8	18.53
9	2	2	2	6	19.68
10	3	1	0	4	10.30
11	3	1	3	7	16.44
12	0	0	1	1	-
Total	47	15	22	84	17.41

The coarse genetic linkage analysis was performed by 46 F_2 individuals of TNG 78 × TCS 17. The recombination frequencies of genotypes of 84 markers and the putative genotypes of *GE2*, which predicted by the segregation ratio of embryo size in approximate 50 F_3 seeds of each F_2 individuals, were analyzed by MAPMAKER/EXP version 3.0 (Lander *et al*. 1987) using the Kosambi function (Kosambi 1944). The *GE2* was resided in the interval of *RM234* and *RM420* on the long arm of chromosome 7, 9.0 cM away from *RM234* and 17.8 cM from *RM420* (Fig. 2).

Fine Genetic Mapping of *Giant Embryo2*

Additional 87 out of 600 F_2 of TNG 78 × TCS 17 planted in the 2nd crop season of 2008 were incorporated with five newly designed polymorphic markers in the target region to narrow down the chromosome segment encompassing *GE2*. *GE2*, flanking by *CH0709* and *CH0720*, is confined in an interval of approximate 170 kb (Fig. 2). The target region of *GE2* is covered by three BACs and encloses 20 genes, 3 putative/hypothetic genes, and one retrotransposon, annotated by MSU Rice Genome Annotation (Osa1) Release 6.1 (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/).

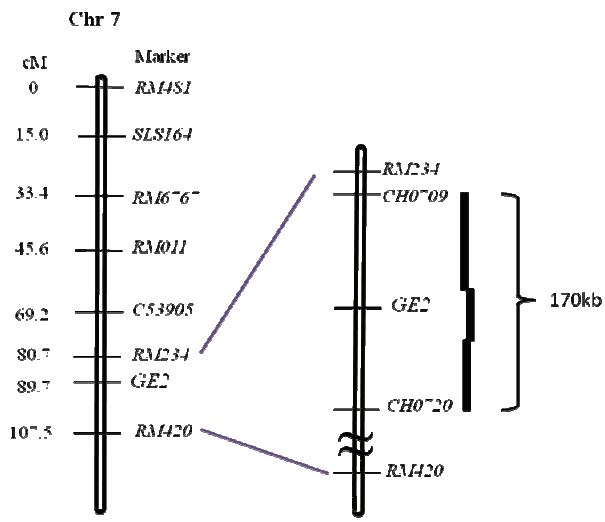

Fig. 2. The genetic and physical maps of *Giant embryo 2* (*GE2*).

DISCUSSION

Giant embryo rice enriched with total proteins, total fatty acids, vitamins, and GABA content in pre-germinated seeds. How to enlarge embryo size is an important task in rice breeding. It is not common to find giant embryo rice from natural germplasm because rice grains with large embryo are usually accompanied with small endosperm, leading to poor germination. Mutagenesis breeding by using chemical mutagens which results few mutations are adopted in giant embryo. A famous Japanese lowland rice cultivar with giant embryo, Hamiminori, was bred by the cross EM40 and Akenohoshi which EM40 was derived from Kinmaze induced by NMU (Hiroshi *et al*. 2001). Several new *indica* and *japonica* varieties were developed by mutagenesis in China, too (Zhang *et al*. 2007; Piao *et al*. 2009). In Taiwan, one of the authors, Y.-P. Wu who is a rice breeder, used NMU to induce TNG 72 and selected from 6 lines exhibiting giant embryo following by the pedigree method. One of six giant embryo mutant lines has 3~5 fold enlarged embryo was claimed as variety TNG 78 in 2009. TNG 78 possesses similar phenotypes of the agronomic traits to its derived parent TNG 72, such as plant height, heading date, yield components, and aroma (Fig. 1). As the high potential nutrition of giant embryo rice, TNG 78 can be utilized in enriching human nutrition as a daily food and as nutrition supplements by brown rice tea bag, healthy nutrition addition agents, and germinated rice.

The locus of *GE2* was coarsely mapped in the interval 26.8 cM, between *RM234* and *RM420* on the long arm of chromosome 7, corresponding to the interval of 14.4 cM from 93.9 cM (*RM234*) and 118.3 cM (*RM420*) on the long arm of chromosome 7, with an interval of 14.4 cM, based on the rice high-density linkage map published by Harushima *et al.* (1998) (Fig. 2). The discrepancy in

interval lengths of *RM234* and *RM420* is because of different numbers of F_2 individuals and different parents used in crosses. The alternative possibility was 46 individuals subjected to coarse mapping were pre-selected for giant embryo phenotype, leading to higher local recombination rates surrounding *GE2*. The locus of *GE* with four alleles was mapped at the interval of 86.7 cM and 93.0 cM of chromosome 7 by means of high resolution mapping and physical mapping (Nagasawa *et al*. 2002). The linkage maps of *GE* and *GE2* are slightly different that *GE2* might be a new locus corresponding to enlarged embryo size.

GE2 was fine mapped in a 170-kb chromosome segment by recombination study of additional 87 F_2 individuals and 5 markers. The candidate genes were delimited to 20 genes and 3 hypothetic/putative genes. Further investigations of high-resolution mapping by more recombinants and high-density markers to confine the target region with less candidate genes, consequently on positional cloning of the isolation gene, are being carried out to elucidate the gene function and regulation of *GE2*. The 'alteration of plant embryo/endosperm size during seed development', including giant embryo *GE* with four alleles, is patented (WO/2007/070687, 2007; US7,582,817 B2, Sep. 1, 2009). Thus, the flanking markers of *GE2*, *CH0709* and *CH0720*, can be implemented to rice breeding programs to breed more varieties with enlarged embryo by marker assisted selection as foreground selection.

In addition, the flanked markers of the giant embryo can be incorporated with the functional marker of golden rice gene *GR1* of *japonica* Tainung 76, a sodium azide derived from *japonica* Tainung 67, to introgress into a purple pericarp line, CNY941201, to promote nutrition of rice which can alleviate malnutrition as rice for the major staple food.

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